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14. ABSTRACT

The monomeric Ral GTPases, RalA and RalB have been recognized as core components of the regulatory framework supporting tumorigenic transformation. Specifically, RalA is required to maintain anchorage independent proliferation while RalB is required to suppress apoptotic checkpoint activation. Here, we have defined the mechanistic contribution of RalB to cancer cell survival. We find that in normal human epithelia, a RalB/Sec5/TBK1 signal transduction cascade connects viral surveillance receptors to activation of host defense gene expression. We find that this pathway is aberrantly engaged by oncogene activation in tumors with the consequence of deflecting programmed cell death pathways that would normally engage in response to oncogene-induced stress. The obligate chronic activation of TBK1 kinase in breast cancer cells coupled with the absence of toxicity upon TBK1 inactivation in normal breast epithelia suggests this protein represents a facile therapeutic target.

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Survival, apoptosis, oncogenic transformation and tumor growth

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FINAL SUMMARY REVIEW
USAMRMC FY03 BREAST CANCER RESEARCH PROGRAM

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SUMMARY REVIEW: Over the past two decades, much attention has been focused on identifying the molecular basis of the participation of oncogenic Ras in tumor initiation and progression. However, activation of the Ral GTPases RalA and RalB has historically been the orphan Ras effector pathway with respect to its role in Ras signaling and oncogenic transformation. Recently, a central and critical role of Ral GTPase activation has come to light in the initiation and maintenance of oncogenic transformation, playing a role in enhanced proliferation and resistance to apoptosis, although little is known about the mechanistic nature of these effects. The main goal of this research is to elucidate the regulatory pathways engaged by RalB to support tumor survival.

In normal human epithelial cells, the endogenous RalB/Sec5/TBK1 activation complex was required for both the Toll-like receptor-dependent and RIG-I-dependent host defense response. Importantly, a central role for Ral and Sec5 in innate immune signaling was genetically validated with observations of a defective antimicrobial response in Drosophila dRal and dSec5 mutants. In human tumor cells, TBK1 was chronically activated as compared to untransformed epithelial cells. The RalB/Sec5/TBK1 activation complex is required for tumor cell survival, but is dispensable in untransformed cells. Consistent with this, *TBK1*-/- MEFS were refractory to oncogenic transformation by Ras12V. Importantly, the RalB/Sec5 complex was both required and sufficient to maintain chronic TBK1 activation and survival in cancer cells and to support TBK1 activation and IRF3-dependent gene expression in response to viral invasion of normal cells.

The central participation of RalB and its effector Sec5 in antiviral surveillance and response pathways (as proximal components of the TBK1 activation machinery) was discovered.

Oncogenic transformation was found to commandeer cell-autonomous host defense signaling to deflect cell-death checkpoint activation, a previously unappreciated but potentially vitally important component of the aberrant regulatory networks supporting tumorigenicity. The mechanistic contribution of RalB to oncogenic transformation was defined. TBK1 was revealed as a candidate drug target with potentially high therapeutic index.

FORMAT/EDITORIAL ISSUES: This final summary does not conform to USAMRMC reporting requirements. The PI has not provided any data (tables and figures) in the body of this report, and the body does not reference any specific figures in the appended articles.

Data presentation should be comprehensive in providing a complete record of research findings for the entire period of the award. Without a comprehensive presentation of data, it is not possible to accurately assess the final progress of this project.

The Conclusions section of this final summary does not summarize the results or include the importance and/or implications of the completed research. It also does not include the "so what" section, which evaluates the knowledge as a scientific or medical product. A more comprehensive Conclusions section is necessary for a final summary.

CONTRACTUAL ISSUES: Information provided in this final summary supports the following:

Task 1	Months 1-12	Completed
Task 2	Months 13-24	Not addressed
Task 3	Months 25-36	Completed

The statements from the PI in the body section indicate that Tasks 1 and 3 are completed, but no data or research description was provided to justify the claim.

Task 2, regarding the effectors of RalA on growth of breast cancer cell lines, was not discussed in this report.

TECHNICAL ISSUES: None.

P.I.'s Response to Review:

Point 1: **FORMAT/EDITORIAL ISSUES:** This final summary does not conform to USAMRMC reporting requirements. The PI has not provided any data (tables and figures) in the body of this report, and the body does not reference any specific figures in the appended articles.

This fellowship supported the work of Yuchen Chien, and then upon her graduation, some of the doctoral work of Ms. Tzuling Chen. The reassignment of support was performed in coordination with the DoD. Below, I reference the figures in the appended articles that support the descriptions above:

In normal human epithelial cells, the endogenous RalB/Sec5/TBK1 activation complex was required for both the Toll-like receptor-dependent and RIG-I-dependent host defense response [Figure 6 panels A-J; Chien et al. Cell 127: 157-170]. Importantly, a central role for Ral and Sec5 in innate immune signaling was genetically validated with observations of a defective antimicrobial response in Drosophila dRal and dSec5 mutants [Figure 6 K; Chien et al. Cell 127: 157-170 . In human tumor cells, TBK1 was chronically activated as compared to untransformed epithelial cells [Figure 7G; Chien et al. Cell 127: 157-170]. The RalB/Sec5/TBK1 activation complex is required for tumor cell survival, but is dispensable in untransformed cells [Figures 1-3; Chien et al. Cell 127: 157-170]. Consistent with this, *TBK1*-/- MEFS were refractory to oncogenic transformation by Ras12V [Figure 7A-E; Chien et al. Cell 127: 157-170]. Importantly, the RalB/Sec5 complex was both required and sufficient to maintain chronic TBK1 activation and survival in cancer cells and to support TBK1 activation and IRF3-dependent gene expression in response to viral invasion of normal cells [Figure 4; Chien et al. Cell 127: 157-170].

Point 2: The Conclusions section of this final summary does not summarize the results or include the importance and/or implications of the completed research. It also does not include the "so what" section, which evaluates the knowledge as a scientific or medical product. A more comprehensive Conclusions section is necessary for a final summary.

Response: As we describe above, these results highlight TBK1 as a conceptually ideal drug target predicted to have high efficacy in cancer cells and little toxicity in normal epithelia. Based on this manuscript, major pharmaceutical companies have begun drug discovery programs targeting TBK1.

Point 3: The statements from the PI in the body section indicate that Tasks 1 and 3 are completed, but no data or research description was provided to justify the claim.

Response: My belief is that the appended reprints justify the claim.

Point 4: Task 2, regarding the effectors of RalA on growth of breast cancer cell lines, was not discussed in this report.

Response: This task was mired in technical difficulties due to the absence of effective antibodies for these effectors. The advertised commercial antibodies proved to be useless, and we are currently generating new ones ourselves.

Cell

RalB GTPase-Mediated Activation of the IκB Family Kinase TBK1 Couples Innate Immune Signaling to Tumor Cell Survival

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SUMMARY

The monomeric RalGTPases, RalA and RalB are recognized as components of a regulatory framework supporting tumorigenic transformation. Specifically, RalB is required to suppress apoptotic checkpoint activation, the mechanistic basis of which is unknown. Reported effector proteins of RalB include the Sec5 component of the exocyst, an octameric protein complex implicated in tethering of vesicles to membranes. Surprisingly, we find that the RalB/Sec5 effector complex directly recruits and activates the atypical IκB kinase family member TBK1. In cancer cells, constitutive engagement of this pathway, via chronic RalB activation, restricts initiation of apoptotic programs typically engaged in the context of oncogenic stress. Although dispensable for survival in a nontumorigenic context, this pathway helps mount an innate immune response to virus exposure. These observations define the mechanistic contribution of RalGTPases to cancer cell survival and reveal the RalB/Sec5 effector complex as a component of TBK1-dependent innate immune signaling.

INTRODUCTION

Much attention has focused over the past two decades on identifying the molecular basis of the participation of oncogenic Ras in tumor initiation and progression. The proximal participation of Ras proteins in the activation of Raf family and PI3K family kinases has been well established together with the mechanistic participation of these Ras effectors in normal and aberrant regulatory networks (Downward, 2003; Mitin et al., 2005; Repasky et al., 2004). However, activation of RalGTPases has historically been the orphan Ras effector pathway with respect to a functional and mechanistic understanding of its role in Ras signaling and oncogenic transformation (Camonis and White, 2005; Feig, 2003). The RalGTPases, RalA and RalB, are close relatives of the founding members of the Ras GTPase superfamily. They are engaged in response to a broad variety of mitogenic, trophic, and hormonal signals by a diverse group of guanyl nucleotide exchange factors that fall into two major groups: those that are directly Ras-responsive via a carboxyterminal Ras binding domain and those that are apparently mobilized by phosphoinositide second messengers via a carboxyterminal pleckstrin homology domain (Feig, 2003).

Recently, a confluence of observations has exposed a central and critical contribution of RalGTPase activation to the initiation and maintenance of oncogenic transformation. Genetically defined human cell models of tumor progression suggest that RalGTPase activation is an essential component of oncogenic Ras-induced transformation in a broad variety of human epithelial cells and is sufficient to drive tumorigenic phenotypes in some backgrounds (Hamad et al., 2002; Rangarajan et al., 2004). RNAi-mediated loss-of-function analysis demonstrated that RalA and RalB collaborate to both support enhanced proliferative propensity and restrict cell-death programs (Chien and White, 2003). Finally, genetic ablation of RalGDS, a Ras-responsive guanyl nucleotide exchange factor, reduced the incidence and progression of topical carcinogen-induced dermal papillomas (Gonzalez-Garcia et al., 2005).

Several candidate and bona fide RalGTP effector proteins have been identified that implicate the participation of these proteins in a bewildering array of dynamic cell biological processes. These include a protein with Rac/CDC42 GAP activity, RalBP1/RLIP, that may participate

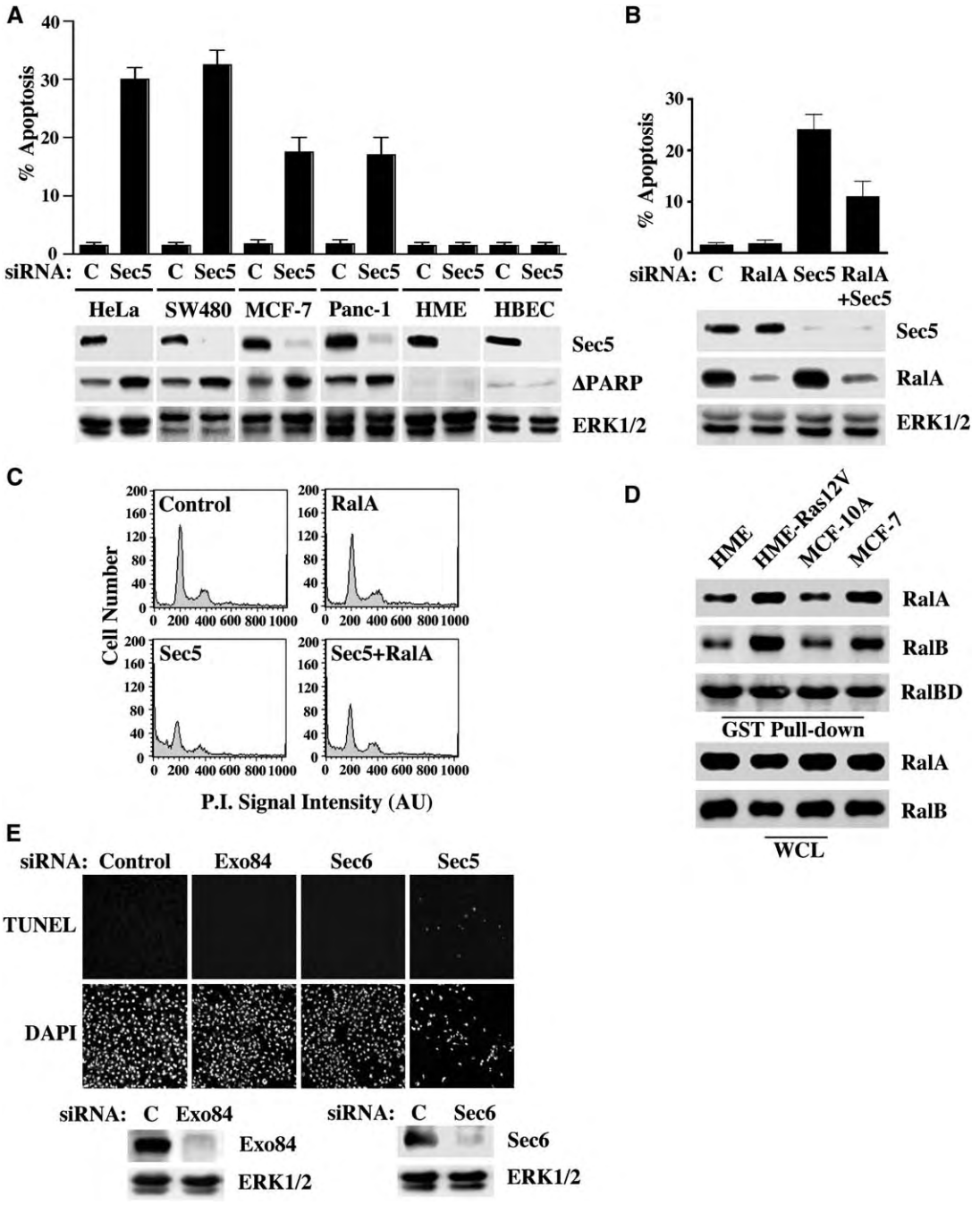

Figure 1. Sec5 is the RalB Effector Supporting Human Tumor Cell Survival

(A) Sec5 deficiency is selectively toxic in cancer cell lines. The indicated human cell lines were transfected with a targetless control siRNA (C) or siRNA targeting human Sec5. Apoptotic cells were scored 72 hr posttransfection and are represented as a percentage of the total cell population ± SD from three independent experiments. The presence of cleaved poly(adenosine diphosphate-ribose)polymerase (ΔPARP) from parallel experiments is shown beneath the graph as an indication of relative caspase activity. Efficacy of RNAi was verified by immunoblots of whole-cell lysates with anti-Sec5 antibodies and ERK1/2 are shown as loading controls. Identical results were observed with an independent siRNA targeting Sec5.

(B) RalA depletion desensitizes cells to loss of Sec5. HeLa cells were transfected with the indicated siRNAs and apoptosis was scored by TUNEL labeling 72 hr posttransfection. Error bars represent mean ± SD of three independent experiments.

(C) An aliquot of cells from (B) was stained with propidium iodide and DNA content was analyzed by FACS. Apoptotic cells appear as a population with sub-G1 signal intensities as a consequence of DNA fragmentation. Representative results from three independent experiments are shown.

(D) Transformed cells display elevated RalGTPase activation. GST-Sec5rbd was used to immobilize endogenous GTP-bound RalA and RalB from the indicated cell lines.

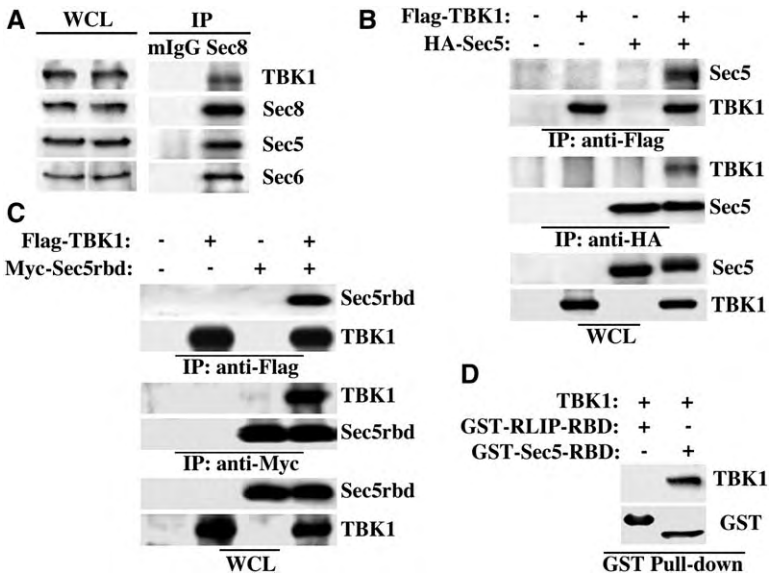

Figure 2. TBK1 Is a Sec5-Interacting Protein

(A) Endogenous TBK1 associates with the exocyst. Anti-Sec8 immunoprecipitates from human bronchial epithelial cells (HBECs) were immunoblotted for the presence of endogenous TBK1 and representative exocyst subunits. Normal mouse IgG (mIgG) was used as a negative control.

(B) TBK1 interacts with Sec5. 293 cells were transfected as indicated and I.P.s were blotted for the presence of FLAG-TBK1 and HA-Sec5.

(C) The minimal Ral-binding domain of Sec5 is sufficient for TBK1 association. The indicated I.P.s were blotted for the presence of FLAG-TBK1 and Myc-Sec5rbd.

(D) Sec5rbd interacts directly with TBK1 in vitro.

together with Ral to regulate a subset of endocytic pathways; filamin, a structural protein that may participate in Ral-dependent filopodia formation; and the exocyst subunits Sec5 and Exo84 that mediate Ral regulation of dynamic secretory vesicle targeting and tethering processes (Camonis and White, 2005; Feig, 2003; Moskalenko et al., 2003).

Here, we have directly investigated the relative contribution of RalGTPase effector proteins to Ral function in normal and tumorigenic human epithelial cells in order to define the regulatory pathway directly engaged by RalB to support tumor cell survival. Surprisingly, we find that a RalB/Sec5 effector complex specifically supports the prosurvival arm of tumorigenic regulatory networks by directly recruiting and activating the atypical IκB kinase family member TBK1 (Tank binding kinase 1). While this process is physically tethered to the exocyst complex, it may be distinct from the participation of RalB or Sec5 in exocyst assembly or function. We show that TBK1 kinases are chronically activated in a variety of cancer cell lines, are required to maintain cancer cell survival, and are required to support oncogenic Ras-induced transformation. Although dispensable for survival of nontumorigenic human epithelial cells in culture, this pathway helps mount an innate immune response to dsRNA or Sendai virus exposure. Therefore, the RalB/Sec5 effector complex is a component of host defense signaling, acting directly at the level of TBK1 activation. These observations define the direct mechanistic contribution of RalB to oncogenic transformation, expose a central component of the proximal machinery required to engage TBK1 in response to antiviral surveillance systems, and reveal the contribution of components of cell-autonomous innate immune pathways to the generation of a tumorigenic regulatory framework.

RESULTS

Sec5 Is the RalB Effector Restricting Apoptosis in Cancer Cells

To define the molecular nature of the RalB effector pathway supporting cancer cell survival, we first carried out an RNAi-based candidate screen of RalGTPase effector proteins. Our expectation was that depletion of the bona fide effector protein supporting this pathway would phenocopy RalB depletion: selective induction of apoptosis in cancer cells versus nontumorigenic epithelial cells (Chien and White, 2003). While depletion of the Ral effectors Exo84 (Moskalenko et al., 2003) or RLIP/RalBP1 (Cantor et al., 1995; Jullien-Flores et al., 1995; Park and Weinberg, 1995) did not uncover any overt contribution of these proteins to cell proliferation or survival (data not shown), depletion of the Ral effector Sec5 (Moskalenko et al., 2002; Sugihara et al., 2002) with multiple independent siRNA duplexes induced apoptosis in a variety of human tumor-derived cell lines (Figure 1A). In contrast, Sec5 was not limiting for survival of normal nontumorigenic telomerase-immortalized human mammary epithelial cells (HME) or bronchial epithelial cells (HBEC; Figure 1A). However, as we previously observed upon depletion of RalB (Chien and White, 2003), depletion of Sec5 did sensitize HME cells to apoptosis in response to a 16 hr release from matrix association (35% of the population as compared to 9.5% in control groups). We have previously observed that RalA-dependent pathological proliferative pressure is offset by RalB-dependent inhibition of

(E) Sec5 supports cell survival independently of exocyst function. HeLa cells were transfected with the indicated siRNAs. Ninety-six hours posttransfection, cells were labeled with TUNEL and counterstained with DAPI as shown. Representative results from three independent experiments are shown.

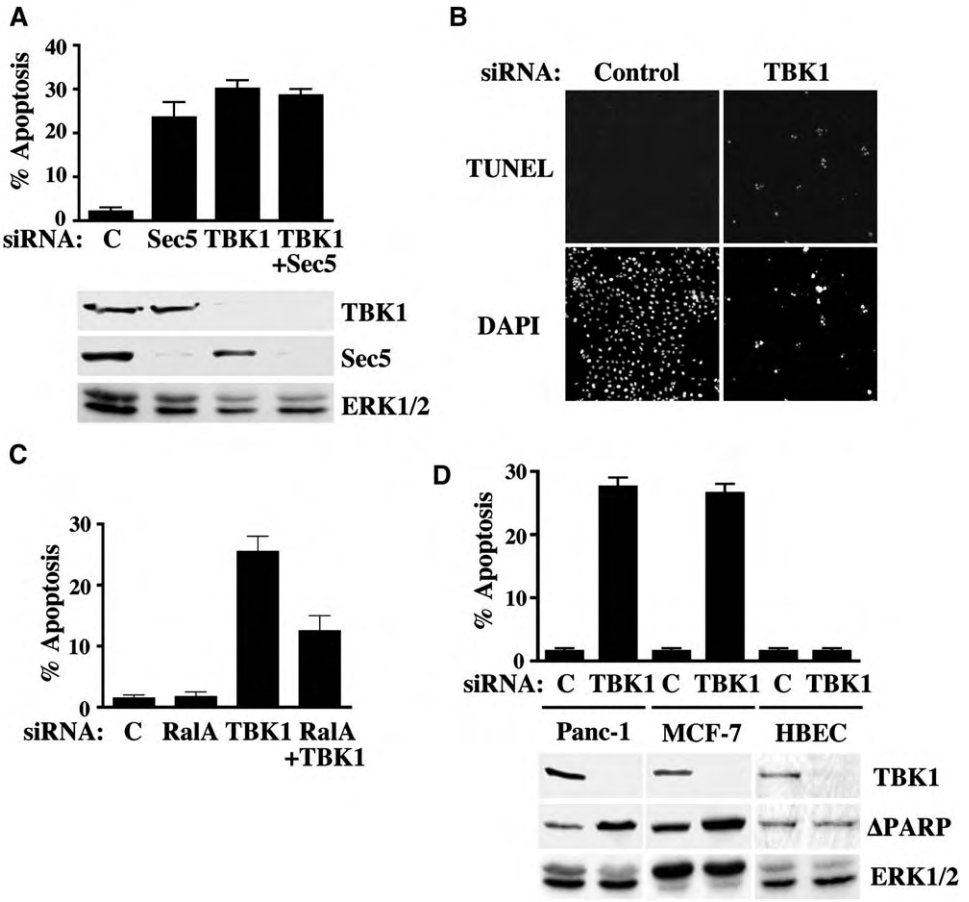

Figure 3. TBK1 Depletion Mimics RalB and Sec5 Depletion

(A) TBK1 deficiency triggers apoptosis. HeLa cells were transfected with the indicated siRNAs. Apoptosis was assayed by TUNEL labeling 72 hr post-transfection. Error bars represent mean ± SD of three independent experiments.

(B) A highly penetrant cell-death phenotype results from long-term TBK1 depletion. HeLa cells were transfected as in (A) except that apoptosis was scored by TUNEL-labeling 120 hr posttransfection. Representative results from three independent experiments are shown.

(C) RalA depletion desensitizes cells to loss of TBK1. The apoptotic response to the indicated siRNAs was evaluated as in (A).

(D) Selective dependence of tumorigenic cell lines on TBK1 for survival. The indicated cell lines were assessed as in (A).

apoptosis (Camonis and White, 2005; Chien and White, 2003). Consistent with this observation, we find higher relative amounts of activated RalA and RalB in transformed cell lines relative to related nontumorigenic cell lines (Figure 1D). As a result of this yin-yang relationship, depletion of RalA relieves the dependency of cancer cells on RalB-dependent survival signaling (Chien and White, 2003). Importantly, depletion of RalA also reduced the sensitivity of tumor cells to loss of Sec5 (Figures 1B and 1C). The observation that Sec5 depletion mimics RalB depletion in multiple backgrounds strongly implicates Sec5 as the immediate downstream effector supporting RalB survival signaling.

Sec5 is a member of the heterooctameric Sec6/8 complex, a.k.a. the exocyst, responsible for regulated targeting and tethering of selective secretory vesicles to specialized dynamic plasma membrane domains (Guo et al., 2000). The sum of observations to date suggest that RalGTPases regulate a Sec5-Exo84 assembly interface for dynamic control of exocyst function in mammalian cells (Moskalenko et al., 2002, 2003). The consequences of Sec5 depletion described above imply a role for secretory machinery in the maintenance of cancer cell survival. However, in contrast to Sec5, depletion of the exocyst subunits Sec6 or Exo84 did not impair cell viability (Figure 1E), suggesting that the contribution of Sec5 to cancer cell survival may be in addition to but separate from its role as a component of the exocyst.

TBK1 Is a Sec5-Interacting Protein Supporting Cancer Cell Survival

To generate leads for discovery of the mechanistic basis of Sec5-dependent cancer cell survival, we examined the molecular composition of the exocyst complex in human epithelial cells. The endogenous exocyst was immunoisolated using monoclonal α-Sec8 antibodies that were

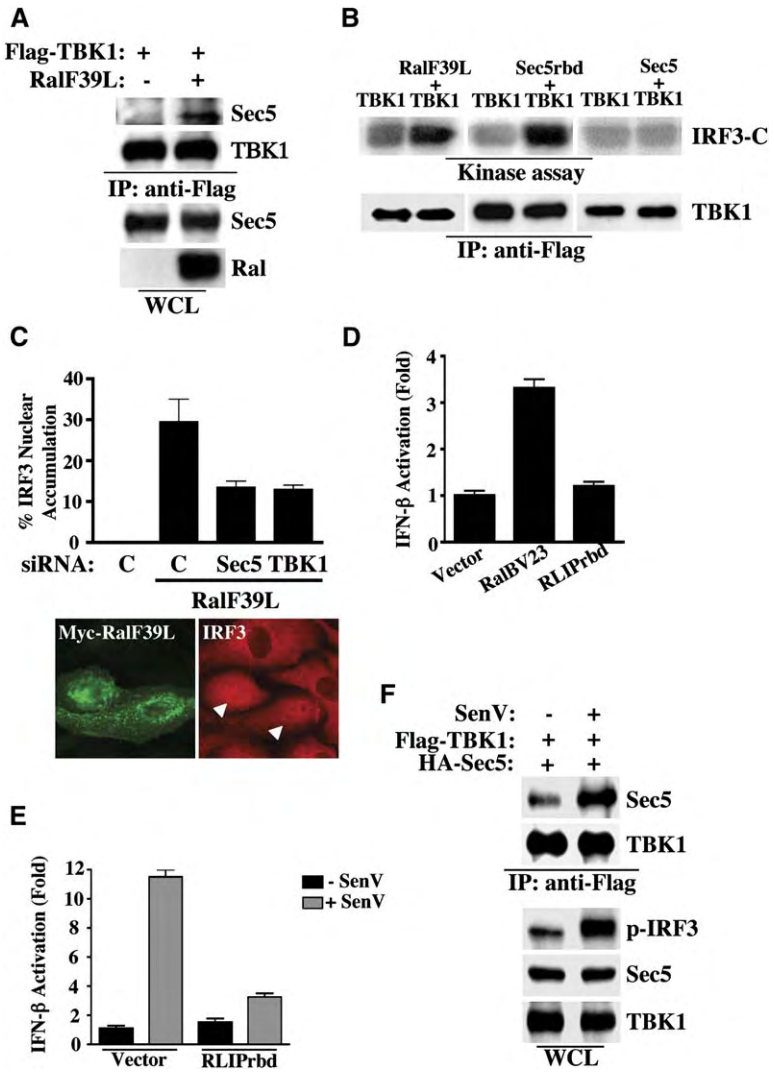

Figure 4. Ral Induces TBK1 Kinase Activation

(A) Ral activation promotes assembly of Sec5/TBK1 complexes. FLAG-TBK1 expressed alone or together with Myc-RalF39L was immunoprecipitated from 293 cells and assayed for associated endogenous Sec5 as indicated.

(B) Both Ral and Sec5rbd are sufficient to trigger TBK1 activation. FLAG-TBK1 expressed alone or together with Myc-RalF39L, Myc-Sec5rbd, or HA-Sec5 was immunoprecipitated from 293 cells and kinase activity was evaluated in vitro using recombinant IRF-3-C as the substrate. ^{32}P incorporation as visualized by phosphorimager analysis following SDS-PAGE is shown. Relative amounts of immunoprecipitated TBK1 are shown.

(C) Ral activation of IRF-3 is Sec5 and TBK1 dependent. HBECs were transfected with Myc-RalF39L together with the indicated siRNAs. Seventy-two hours posttransfection, endogenous IRF-3 localization in Ral-transfected cells was examined by immunofluorescence. Error bars represent mean ± SD of three independent experiments. Representative cells transfected with RalF39L are shown. Nuclear accumulation of endogenous IRF-3 is indicated by the arrowheads.

(D) Ral is sufficient to activate the IFN-β promoter. Osteosarcoma cells were cotransfected with CMV promoter regulated *Renilla* luciferase, and IFN-β promoter regulated firefly luciferase reporter together with the indicated constructs. Cells were incubated for 24 hr, harvested, and extracts subjected to a standard dual luciferase assay. Bars indicate relative IFN-β luciferase activity ± SD.

(E) Ral is required for Sendai virus-induced activation of the IFN-β promoter. Cells were treated as in (D) except with an additional 20 hr incubation in the presence or absence of Sendai virus (100 HA/ml). Bars indicate relative IFN-β luciferase activity ± SD.

(F) Viral infection promotes assembly of Sec5/TBK1 complexes. FLAG-TBK1 was immunoprecipitated from HBEC cells and assayed for associated Sec5 as indicated 5 hr postinfection with Sendai virus (100 HA/ml).

previously shown to effectively precipitate the full octameric complex (Grindstaff et al., 1998). The proteins in the complex were identified by nano-HPLC electrospray ion trap mass spectrometry following 1D gel chromatography (Zhao et al., 2004a). From each of three independent analyses of complexes recovered from the triton-soluble fraction of cell lysates, we identified the exocyst components Sec8, Sec3, Sec5, Sec6, and Sec10 together with the atypical IκB kinase (IKK) family member TBK1/NAK (Tank binding kinase 1/NFκB activating kinase). The presence of TBK1 in the exocyst complex was validated by immunoblot analysis of α-Sec8 immunoprecipitates (Figure 2A).

While TBK1$^{-/-}$ mouse embryo fibroblasts proliferate normally in culture, genetic ablation of TBK1 is embryonic lethal, suggesting TBK1 supports cell survival during development (Bonnard et al., 2000). TBK1 has recently been recognized as a central component of the regulatory network supporting host defense responses. TBK1 can directly phosphorylate both the p65 Rel and IRF-3 transcription factors, enhancing their transactivating potential, and is required for IRF-3 activation in response to viral invasion (Buss et al., 2004; Fitzgerald et al., 2003; McWhirter et al., 2004; Sharma et al., 2003). Intriguingly, both IRF-3 and its close homolog IRF-7 have been implicated in promotion of oncogenic transformation (Lubyova et al., 2004; Zhang et al., 2004). Given these relationships, we investigated the possible contribution of TBK1 to RalB/Sec5-dependent survival signaling. In vitro binding assays, using recombinant TBK1 together with individual exocyst subunits

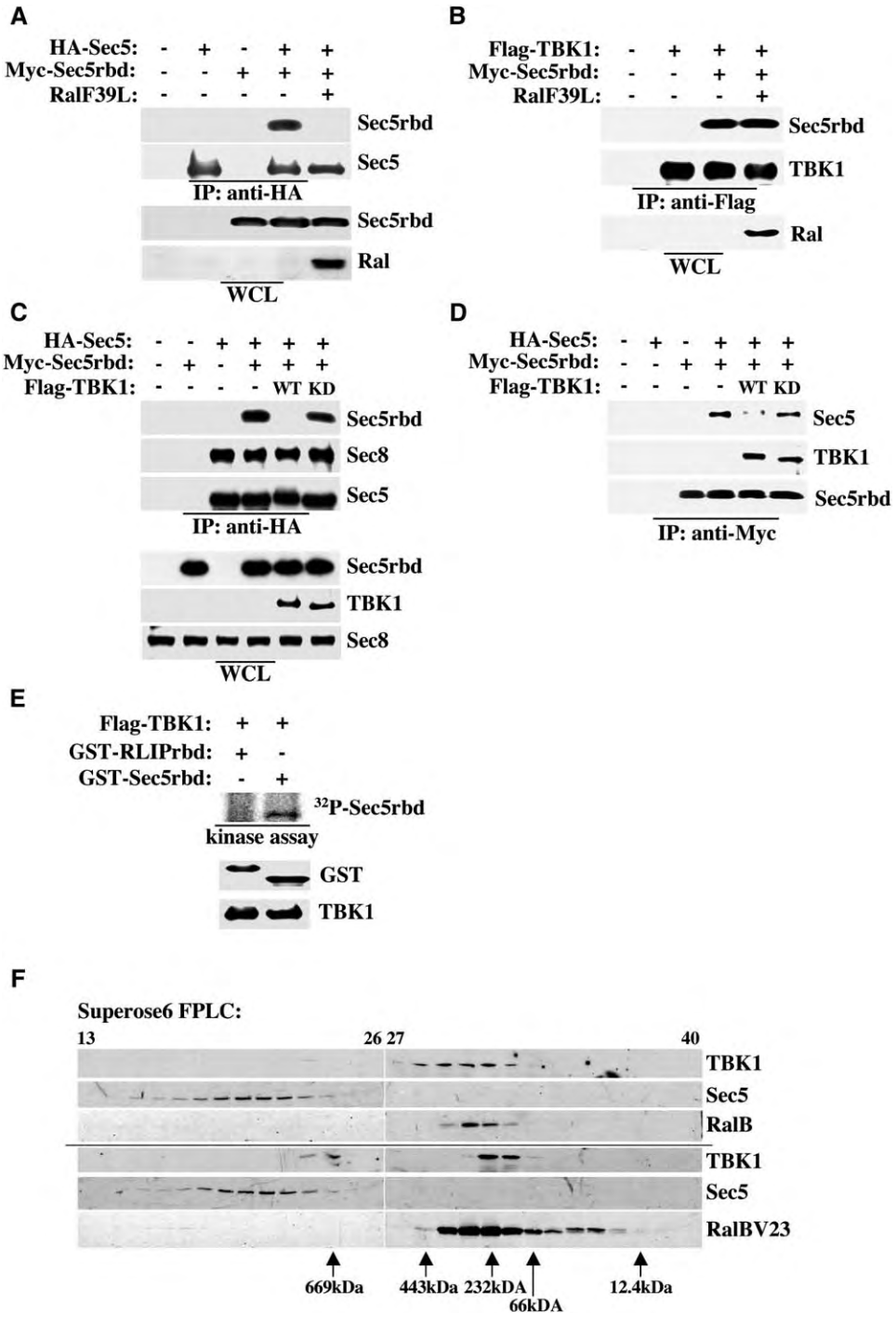

Figure 5. Ral Binding Exposes a Sec5/TBK1 Interaction Interface

(A) Active Ral blocks Sec5 dimerization. 293 cells were transfected with the indicated plasmids. Forty-eight hours posttransfection, HA-Sec5 was immunoprecipitated and assayed for coprecipitating Myc-Sec5rbd in the presence or absence of RalF39L expression.

(B) Ral and TBK1 do not compete for Sec5rbd binding. FLAG-TBK1 was immunoprecipitated and assayed for coprecipitating Sec5rbd in the presence or absence of RalF39L expression.

(C) Kinase-domain-dependent inhibition of Sec5 dimerization by TBK1. The association of HA-Sec5 with Myc-Sec5rbd and endogenous Sec8 was examined in the presence or absence of wild-type FLAG-TBK1 (WT) or kinase-dead FLAG-TBK1-K38A (KD) expression.

(D) Both wild-type and kinase-dead TBK1 bind Sec5rbd. The presence of the indicated proteins was examined in Myc-Sec5rbd immunoprecipitates.

(E) TBK1 phosphorylates Sec5rbd. FLAG-TBK1 expressed in 293 cells was immunoprecipitated and incubated with purified recombinant GST-RLIPrbd or GST-Sec5rbd together with ^{32}P-ATP.

produced in rabbit reticulocyte lysates, identified Sec5 as the TBK1-interacting exocyst subunit (data not shown). This was validated with reciprocal coimmunoprecipitation of epitope-tagged TBK1 and Sec5 from HEK293 cells (Figure 2B). Truncation analysis identified the amino terminus of Sec5 as minimally sufficient for TBK1 interaction (Figure 2C). This region completely overlaps with the previously defined Ral-binding domain of Sec5 (Sec5rbd, amino acids 1–120) that directly interacts with activated RalGTPases (Moskalenko et al., 2002; Sugihara et al., 2002). Sec5rbd also directly interacted with TBK1 in vitro (Figure 2D); however, this interaction is not a common feature among "Ral-binding domains," as a distinct minimal Ral-binding domain from the candidate Ral effector RalBP1/RLIP did not bind TBK1 (Figure 2D).

We examined the potential functional relevance of TBK1/Sec5 complexes through siRNA-mediated loss of function. Mimicking the consequences of RalB or Sec5 deficiency, cells depleted of TBK1 engaged an asynchronous apoptotic program, such that approximately 25% of the population scored positive for apoptotic markers at any one time (Figure 3A) and the majority of the cells were dead by 150 hr posttransfection (Figure 3B). Codepletion of Sec5 together with TBK1 did not appreciably alter the apoptotic response as compared to depletion of either alone, implying action on a common pathway (Figure 3A). Consistent with a functional interaction of TBK1 with RalB and Sec5, this phenotype was rescued by codepletion of RalA (Figure 3C). Finally, like RalB and Sec5, TBK1 is a limiting factor for survival of a variety of tumor-derived cell lines but not nontumorigenic epithelial cells (Figure 3D).

RalB Activates TBK1 by Promoting Sec5/TBK1 Complex Assembly

The portion of Sec5 participating in both RalGTP and TBK1 binding is an immunoglobulin-like structure similar to the NFκB p50 dimerization domain that falls into the IPT/TIG family of protein domains (Fukai et al., 2003). Given that GTP-bound RalB and TBK1 directly interact with the same domain in Sec5, we examined the consequences of RalGTPase regulation on the Sec5/TBK1 complex and TBK1 kinase activity. Expression of a "fast exchange" variant of Ral that constitutively activates Ral regulatory pathways in cells without grossly interfering with exocyst function (Moskalenko et al., 2002) promoted TBK1 association with endogenous Sec5 (Figure 4A). This indicates that, in contrast to potential steric hindrance, Ral activation drives TBK1/Sec5 complex assembly. Importantly, Ral-induced TBK1/Sec5 complex formation correlated with enhanced TBK1 kinase activity as measured by in vitro phosphorylation of recombinant IRF-3, a direct TBK1 substrate (Fitzgerald et al., 2003; Sharma et al., 2003; Figure 4B). Surprisingly, expression of the Sec5rbd, which is minimally sufficient for direct TBK1 interaction, was also sufficient to enhance TBK1 kinase activity. In contrast, expression of full-length Sec5 had no impact on TBK1 activation (Figure 4B). As mentioned above, a direct downstream consequence of TBK1 activation is phosphorylation of the cytoplasmic IRF-3 transcription factor. This modification induces IRF-3 dimerization and nuclear translocation to trigger interferon-response gene-expression programs (Fitzgerald et al., 2003; Sharma et al., 2003). Consistent with a RalB/Sec5/TBK1 regulatory cascade, expression of activated Ral in normal human bronchial epithelial cells was sufficient to induce Sec5- and TBK1-dependent nuclear translocation of endogenous IRF-3 (Figure 4C). In further support of this relationship, RalB was sufficient to activate expression of a luciferase reporter gene driven by the IRF-3-responsive Interferon-β (IFN-β) promoter (Figure 4D; Foy et al., 2003). In addition, Sendai virus-induced activation of the IFN-β promoter was severely abrogated upon inhibition of endogenous Ral protein function by expression of a dominant interfering RalGTP binding domain from RLIP/RalBP1 (RLIPrbd [Moskalenko et al., 2002]; Figure 4E). Finally, we found that Sec5/TBK1 complexes accumulate in response to host defense pathway activation by Sendai virus infection (Figure 4F).

The differential capacity of Sec5rbd versus full-length Sec5 to induce TBK1 activation, coupled with our previous observations that Sec5 can self-associate (Formstecher et al., 2005), suggested the possibility that RalB may promote Sec5/TBK1 association and TBK1 activation by relieving autoinhibitory interactions within the Sec5 protein. We found that Sec5 and Sec5rbd interacted robustly in cells and that activated Ral could effectively block this interaction (Figure 5A). In contrast, expression of activated Ral did not detectably modulate the interaction between TBK1 and Sec5rbd (Figure 5B). This suggests that while RalB and TBK1 have independent interaction interfaces on Sec5rbd, RalB can sterically hinder Sec5 self-association. Intriguingly, the Sec5/Sec5rbd interaction was also blocked by expression of TBK1 but in a manner that was dependent on TBK1 kinase activity (Figure 5C). Both wild-type and kinase-dead TBK1 coprecipitated equivalently well with Sec5rbd (Figure 5D), indicating that the capacity of TBK1 to dissociate Sec5/Sec5rbd complexes was not a consequence of competitive interactions. However, TBK1 can directly phosphorylate Sec5rbd (Figure 5E), raising the possibility that TBK1 kinase activity may stabilize an "open conformation" of Sec5—possibly facilitating feedback amplification of TBK1 activation. The cumulative relationships described above suggest a two-step model for Ral-induced TBK1 activation, in which RalB/Sec5 complex formation reveals a cryptic TBK1-interaction domain that recruits TBK1 into a kinase activation complex. Consistent with the capacity of RalB to modulate

(F) RalB activation modulates endogenous TBK1 complexes. MDCK cells were transfected with vector (top panels) or RalB23V (bottom panels). Forty-eight hours posttransfection, cleared lysates were fractionated by FPLC. Fractions 12–40 were immunoblotted with the indicated antibodies. Molecular weight standards eluted at the indicated fractions.

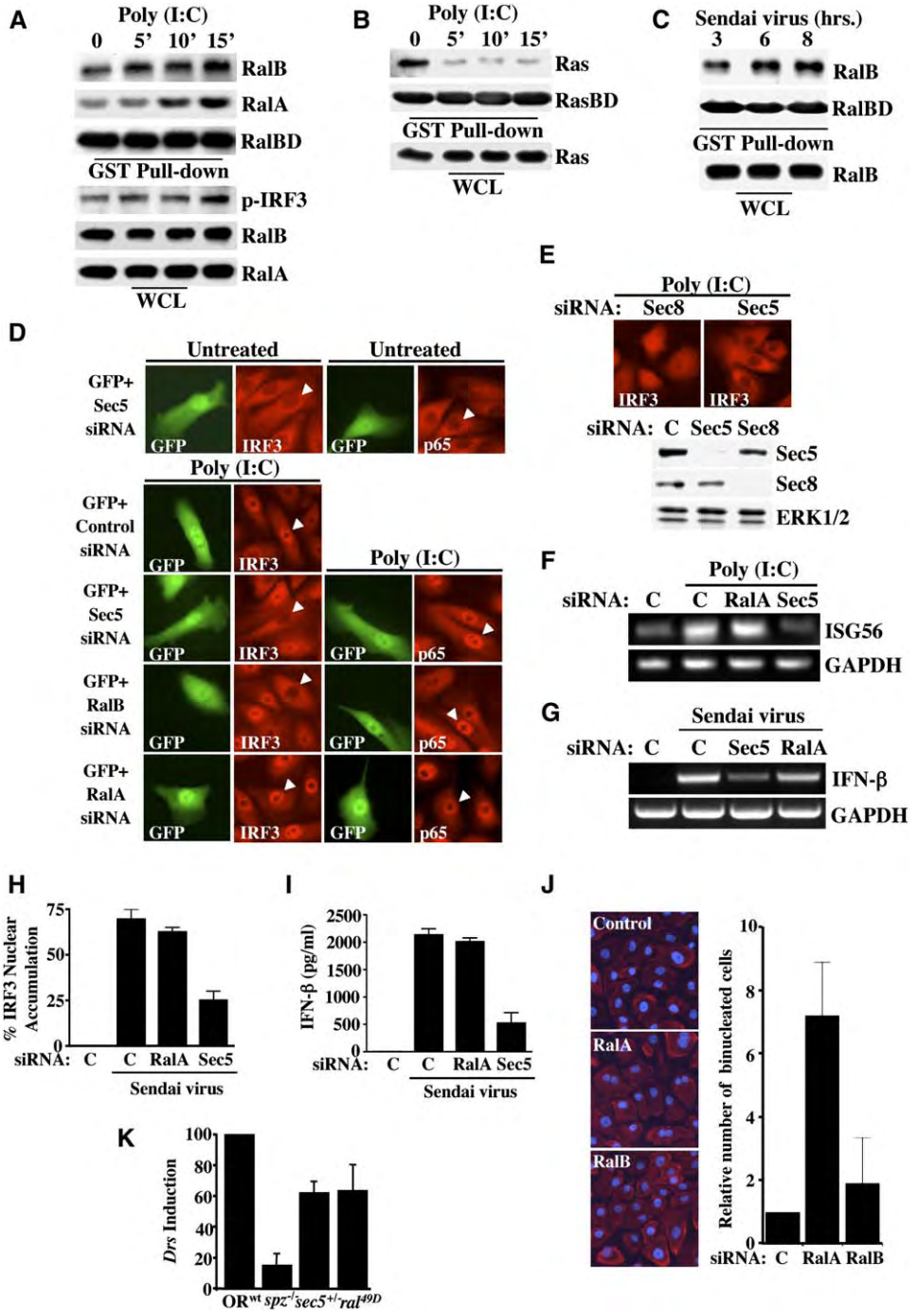

Figure 6. RalB and Sec5 Are Required for Host Defense Pathway Activation

(A) RalGTPases are activated in response to TLR3 signaling. HBECs were treated with poly(I:C) (100 μg/ml) for the indicated time points (0, 5, 10, 15 min). GST-Sec5rbd was used to immobilize endogenous GTP-bound RalA and RalB. Phospho-IRF-3 (Ser396) from whole-cell lysates (WCL) is shown to indicate responsiveness to poly (I:C).

(B) RasGTPases are inactivated in response to TLR3 signaling. HBECs were treated as in (A). GST-Raf1rbd was used to immobilize endogenous GTP-bound Ras.

(C) RalB is activated in response to Sendai virus infection. HBECs were exposed to Sendai virus (100 HA/ml) for the indicated times and assessed for RalB activation as in (A).

(D) RalB and Sec5 are required for IRF-3 but not p65 responsiveness to TLR3 activation. HBECs were cotransfected with a plasmid expressing GFP together with the indicated siRNAs. This method delivers RNA and DNA into a small subset of the population, which can be monitored by GFP expression and allows observation of potentially defective responses within a background of appropriately responding cells. Seventy-two hours

dynamic TBK1 protein/protein interactions, we found that RalB activation was sufficient to promote the formation of high molecular weight TBK1 complexes, as assessed by size exclusion chromatography that cofractionated with a subpopulation of Sec5 in MDCK cells (Figure 5F). These combined observations indicate a physiologically relevant mechanistic relationship among RalB, Sec5, and TBK1 in which RalB drives TBK1 activation directly through Sec5 to support cancer cell survival.

The RalB/Sec5 Effector Pathway Supports Host Defense Signaling

Both genetic and biochemical analysis has firmly positioned TBK1 as a central component of the innate immune response pathways that connect detection of viral invasion to induction of host defense gene expression programs (Balachandran et al., 2004; Fitzgerald et al., 2003). Given the observation that RalB can promote TBK1 activation through Sec5, we investigated the contribution of RalB and Sec5 to the TBK1-dependent host defense response. Exposure of telomerase-immortalized human bronchial epithelial cells (HBECs) to polyinosine-polycytidylic acid (poly(I:C)), a synthetic mimic of viral dsRNA, resulted in the activation of endogenous RalGTPases within 5–10 min (Figure 6A), suggesting Ral activation is an immediate early response to innate immune signaling triggered by Toll-like receptor 3 (TLR3) and/or MDA5 (Alexopoulou et al., 2001; Kato et al., 2006; Kawai et al., 2005; Lee et al., 2006). On the other hand, Ras activation was suppressed, suggesting dsRNA surveillance couples to Ral activation in a Ras-independent manner (Figure 6B). In addition, exposure of HBECs to Sendai virus induced accumulation of RalB-GTP by 6 hr postinfection (Figure 6C). SiRNA-mediated depletion of RalGTPases and exocyst subunits revealed that poly(I:C)-induced mobilization of IRF-3 nuclear translocation in HBECs was dependent upon endogenous RalB and Sec5. In contrast, RalA and Sec8 were not limiting for this response (Figures 6D and 6E). Poly(I:C)-induced mobilization of p65 NFκB nuclear accumulation is independent of TBK1, bifurcating at the level of TRIF (TIR domain-containing adaptor-inducing IFN; Sato et al., 2003; Yamamoto et al., 2003). Neither RalB nor Sec5 depletion interfered with poly(I:C)-induced p65 nuclear accumulation (Figure 6D). This indicates that in the absence of RalB or Sec5, the dsRNA receptor signaling system is still functional but is selectively impaired for TBK1 activation. Depletion of Sec5, but not RalA, also blocked poly(I:C) induction of endogenous IRF-3-dependent gene expression (Figure 6F). Activation of IRF-3 and induction of type I interferon expression in response to Sendai virus infection was also Sec5 dependent (Figures 6G, 6H, and 6I). The exocyst has recently been implicated as a central component of the vesicle trafficking machinery required for the fission step of cytokinesis in epithelial cells (Gromley et al., 2005). Our group and others have directly implicated RalA in the assembly and function of the exocyst (Camonis and White, 2005; Moskalenko et al., 2002, 2003; Shipitsin and Feig, 2004). In concordance with a distinct separation of function of RalA and RalB in HBECs, depletion of RalA, but not RalB, resulted in a marked accumulation of binucleated cells, presumably resulting from cytokenesis defects (Figure 6J). Our cumulative observations indicate that RalB and Sec5 are direct and obligate components of the TBK1-dependent innate immune mechanism in normal nontumorigenic human epithelial cells. The role of Sec5 is likely in addition to, but distinct from, its occupation as a central exocyst component.

To examine the contribution of Ral and Sec5 to the host defense response in vivo, we examined the Toll pathway-dependent antimicrobial response in *Drosophila*. Homozygous deletions of dRal or dSec5 result in early embryonic lethality (Murthy et al., 2003). However, flies with a single copy of dSec5 or with hypomorphic mutations in dRal are viable. Fungal infection-induced expression of

posttransfection, cells were treated with poly(I:C) as indicated for 90 min. Endogenous IRF-3 or p65 (NF-κB) compartmentalization was examined by immunostaining with the appropriate antibodies. Representative cells from three independent experiments are shown.

(E) HBECs were transfected, using a high-efficiency protocol, with the indicated siRNAs. Seventy-two hours posttransfection, cells were treated as in (D). Endogenous IRF-3 was examined as in (D). Representative cells from three independent experiments are shown.

(F) Sec5 is required to trigger interferon response gene expression. HBECs were transfected as in (E) with the indicated siRNAs then treated with poly(I:C). Total RNA was isolated for RT-PCR using primers specific for ISG56 and GAPDH. Representative results from two independent experiments are shown.

(G) Sec5 is required for viral activation of IFN-β. Cells were treated as in (F) except that poly(I:C) was substituted with a 17 hr exposure to Sendai virus (100 HA/ml). Total RNA was isolated for RT-PCR using primers specific for IFN-β and GAPDH. Representative results from two independent experiments are shown.

(H) Sec5 is required to trigger IRF-3 activation in response to Sendai virus exposure. Seventy-two hours following transfection with the indicated siRNAs, HBECs were exposed to Sendai virus (100 HA/ml) for 17 hr. Endogenous IRF-3 nuclear accumulation was scored following immunostaining as in (D). Error bars represent mean ± SD.

(I) Sec5 is required for IFN-β production in response to Sendai virus exposure. HBECs were treated as in (H). Culture supernatants were collected, cleared by centrifugation, and IFN-β concentrations measured by ELISA. Error bars represent mean ± SD.

(J) RalA deficiency results in defective cytokinesis. HBECs transfected with the indicated siRNAs were labeled with rhodamine-phalloidin and DAPI. The number of binucleated cells in each group was scored and normalized to the nontargeting siRNA control. The relative fold change ± SD is shown together with representative fluorescence micrographs.

(K) dSec5 and dRal participate in the *Drosophila* antimicrobial response. Septic injuries were produced by pricking 3- to 6-day-old male adults of the indicated genotypes (20 animals for each group) with a needle coated with a mixture of Gram-negative bacteria (*Erwinia carotovora carotovora*) and fungi (*Candida albicans*). Ten hours later, total RNA was extracted and evaluated by quantitative real-time RT-PCR for *Drosomycin* (*Drs*) and *ribosomal protein 49* (*rp49*) gene expression. Values indicate *Drs* expression, normalized to *rp49*, relative to the response in wild-type control animals (arbitrarily set at 100). Error bars indicate standard deviation from the mean from three independent experiments.

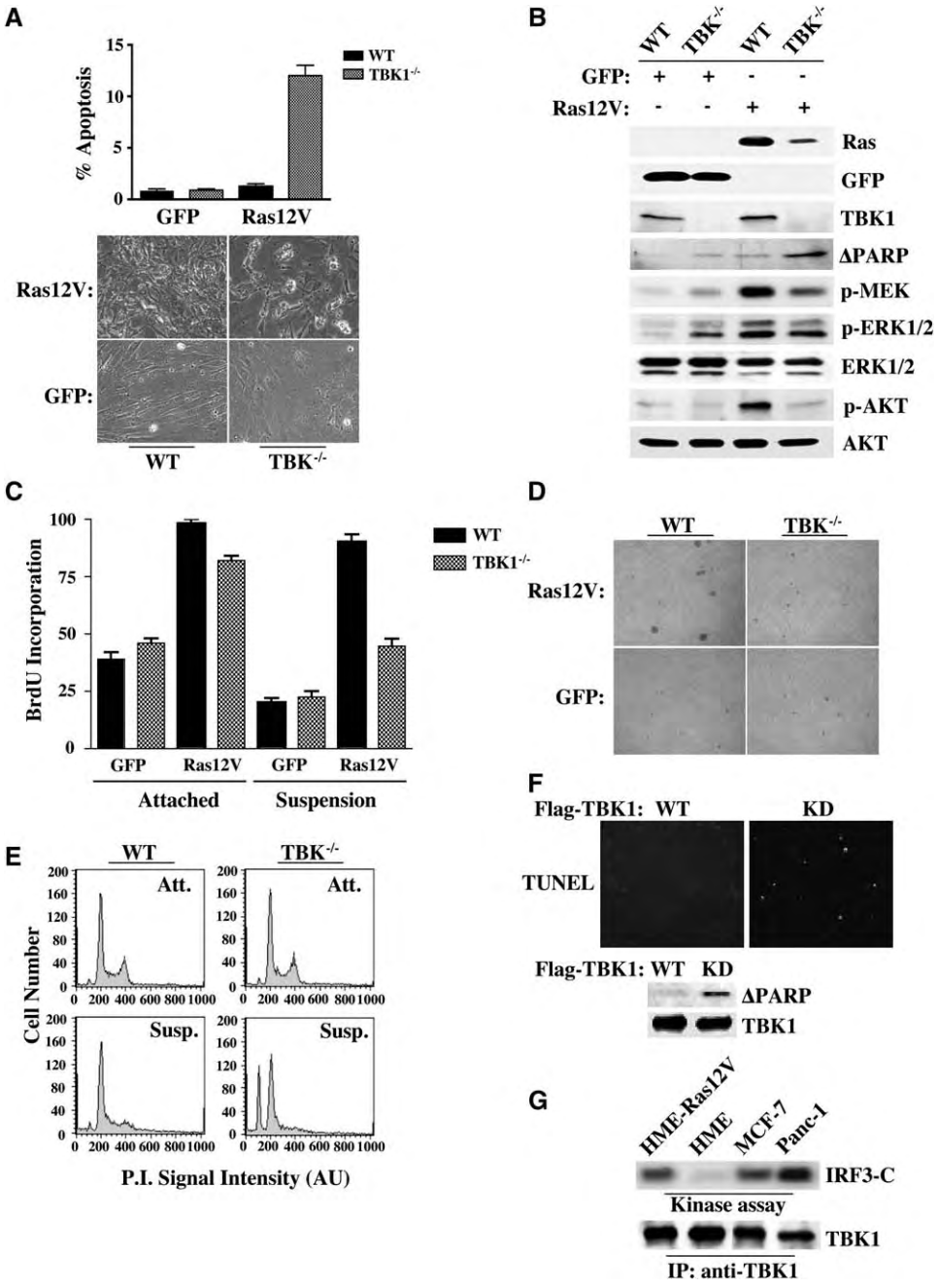

Figure 7. TBK1 Is Required for Oncogenic Ras-Induced Transformation

(A) TBK$^{-/-}$ cells do not tolerate acute oncogenic Ras expression. Wild-type (WT) and TBK1$^{-/-}$ mouse embryonic fibroblasts (MEFs) were infected with replication-defective retrovirus encoding GFP (as a control) or K-Ras12V. Forty-eight hours postinfection, apoptosis was scored by TUNEL. Error bars represent mean ± SD. Representative phase-contrast images of cell monolayer morphology are shown below the graph.

(B) An aliquot of cells from (A) was lysed and analyzed by SDS-PAGE with the indicated antibodies.

(C) Loss of TBK1 impairs oncogenic Ras-induced anchorage-independent proliferation. Forty-eight hours posttransduction with GFP or Ras12V, WT, and TBK$^{-/-}$ MEFs were incubated with BrdU for 17 hr in adherent or suspension cultures. BrdU incorporation is represented as a percentage of the total population. Error bars represent mean ± SD.

(D) Levels of oncogenic Ras expression tolerated by TBK1$^{-/-}$ cells are insufficient to drive colony formation in soft agar. WT and TBK$^{-/-}$ MEFs transduced with GFP or Ras12V as described in (A), were seeded in semisolid agar. After 10 days, numerous small colonies developed in response to Ras expression in the WT MEFS. TBK1$^{-/-}$ cells were unresponsive. Representative fields of view are shown from two independent analyses.

(E) TBK$^{-/-}$ cells are sensitized to anoikis. 20 hr following release from matrix, DNA content of propidium iodide-stained cells was analyzed by FACS. Representative results from three independent experiments are shown.

the *Drs* gene, encoding the antifungal peptide Drosomycin, is a robust readout of Toll pathway activation in *Drosophila* fat body cells (Hoffmann and Reichhart, 2002). As shown in Figure 6K, either haploinsufficiency at the *Sec5* locus or reducing Ral expression with a hypomorphic mutation (Ral^{49D}) blunts the response of fat body cells to fungal invasion. While the observed defects are weak as compared to a homozygous deletion of Spaetzle, the Toll receptor ligand, they were reproducible and consistent with a partial loss of function.

TBK1 Is Required for Oncogenic Transformation

The selective dependence of transformed cells on TBK1 suggests that establishment of a cell-autonomous tumorigenic regulatory network includes commandeering components of innate immune signaling to deflect cell-death checkpoint activation. This relationship predicts that TBK null cells would be refractory to oncogenic transformation. To test this prediction, spontaneously immortalized $TBK1^{-/-}$ mouse embryonic fibroblasts and their wild-type counterparts were infected with retrovirus encoding oncogenic K-ras (K-rasG12V) or GFP. Forty-eight hours postinfection, the majority of the wild-type parental MEFS infected by oncogenic Ras displayed disorganized multilayered growth with the spindle-shaped, refractile morphology typically observed in response to Ras. In contrast, the $TBK^{-/-}$ MEFS accumulated a large number of TUNEL-positive apoptotic cell bodies (Figure 7A). The toxicity associated with retroviral infection of the $TBK^{-/-}$ MEFS was specific to Ras expression, as infection by the identical retroviral backbone driving GFP expression was well tolerated (Figures 7A and 7B). The $TBK^{-/-}$ MEFS surviving oncogenic Ras transduction expressed significantly less ectopic K-ras as compared to the parental cells, suggesting negative selection against high K-ras-expressing cells in the absence of TBK1 (Figure 7B). The amount of tolerated K-ras expression in $TBK^{-/-}$ MEFS only slightly enhanced MEK activation, failed to induce AKT phosphorylation, and was associated with elevated accumulation of cleaved PARP (Figure 7B). While oncogenic K-ras transduction of the parental MEFS was sufficient to support anchorage-independent proliferation and the growth of colonies in soft agar, the amount of K-ras expression in the $TBK^{-/-}$ MEFS only weakly enhanced anchorage-independent proliferation (Figure 7C) and failed to significantly promote colony-formation in soft agar (Figure 7D). In fact, the $TBK^{-/-}$ cells were themselves sensitized to the anoikis response as compared to wild-type controls, much as we have observed upon depletion of RalB or Sec5 in human epithelial cells (Figure 7E). These observations suggest TBK1 expression is required for cells to tolerate transforming levels of oncogenic Ras expression. In further support of this relationship, we found that expression of kinase-dead TBK1 in oncogenic K-ras-expressing MEFs induced apoptosis, presumably through dominant inhibition of native TBK1 activation (Figure 7F). Finally, we find that cancer cell lines (MCF7 and Panc1) and oncogenic Ras transformed mammary epithelial cells (HME-Ras12V) display elevated chronic TBK1 kinase activity as compared to nontumorigenic mammary epithelial cells (HME; Figure 7G).

DISCUSSION

Through elaboration of the molecular components directly mediating RalB-dependent survival signaling in cancer cells, we have identified a novel signal transduction cascade connecting Ral activation to the atypical IKK family kinase TBK1 and the cognate TBK1-dependent transcription factors. We find that normal airway epithelial cells require a RalB/Sec5/TBK1 activation complex to support the host defense response triggered by either extracellular dsRNA or exposure to Sendai virus. Remarkably, this pathway is chronically activated in cancer cells at the level of RalB and is required to support pathological survival in the context of a tumorigenic regulatory environment.

TBK1 is a central node in the regulatory network required to trigger host defense gene expression in the face of a virally compromised environment. This kinase is engaged downstream of multiple sentinel proteins in the host defense response system (Beutler, 2004; Hiscott, 2004; Kato et al., 2006; Kawai et al., 2005; Lee et al., 2006; McWhirter et al., 2005); however, the mechanistic basis of TBK1 activation downstream of these triggers is largely unknown. The adaptor protein TRIF is a TLR3-associated molecule that is required to engage TBK1 activation and can be found in a complex together with TBK1, TRAFs, and IRF-3. However, the interaction of TBK1 with TRIF apparently occurs subsequent to TBK1 activation, indicating the presence of additional unknown factors proximally associated with TBK1 that lead to kinase activation (Sato et al., 2003). One candidate is NAP1 (NAK-associated protein 1), a TANK-related protein that can enhance TBK1 activation in vitro and is required for TLR3/4-activation of the IFN-β promoter (Fujita et al., 2003). Here, we find that the exocyst complex subunit and RalGTPase effector protein Sec5 binds directly to TBK1 and is necessary and sufficient to trigger TBK1 kinase activity in bronchial epithelial cells. This interaction appears to be regulated by RalB-dependent display of the TBK1 interaction interface on the amino terminus of Sec5. Whether the RalB/Sec5/TBK1 activation complex functions independently of TRIF or acts between TRIF and TBK1 remains to be determined. The observation that Sec5 is required for both dsRNA- and SenV-mediated IFN responses suggests that the RalB/Sec5 effector complex may be employed by multiple regulatory systems converging on TBK1 activation (Kato et al., 2006).

(F) Kinase-dead TBK1 expression induces apoptosis in Ras-transformed cells. The indicated constructs were expressed in K-ras transformed MEFs derived from (A) for 48 hr. Apoptosis is indicated by TUNEL and PARP cleavage.

(G) TBK1 kinase activity in normal and transformed cell cultures. Endogenous TBK1 kinase activity was assayed as described in Figure 4B.

Sec5 is an integral component of the Sec6/8 complex, or exocyst. This eight-subunit vesicle targeting machine plays a central role in the generation and maintenance of functionally specified plasma membrane domains (Novick and Guo, 2002). We found that the contribution of Sec5 to TBK1 activation occurs independently of exocyst integrity. This highlights a regulatory role for Sec5 proteins beyond their contribution to dynamic vesicle trafficking. Nevertheless, TBK1 is clearly recruited to the exocyst complex and is likely engaged by Sec5 from within that context. We have not yet determined if there are any fundamental functional relationships between host defense signaling and the exocyst. However, guilt by association would suggest that these two systems are coupled to allow coordinate regulation of multiple dynamic processes that facilitate an effective host defense response. Given the obligatory role of the exocyst in endosomal trafficking steps, localization of Sec5 to endosomal compartments containing antiviral surveillance receptors may represent adoption of a strategic location for generation of antiviral signaling platforms.

We found that TBK1 is required to support oncogenic Ras transformation, and TBK1 kinase activity is elevated in transformed cells and required for their survival in culture. Widespread evidence indicates that apoptosis is often a collateral consequence of pathological regulation of cell-cycle machinery (Abrams and White, 2004). This mechanistic coupling between cell-cycle and cell-death pathways likely represents a key component of the cell-autonomous restraints governing tissue homeostasis (Evan and Vousden, 2001; Green and Evan, 2002). As a consequence, oncogene-driven aberrations in the regulatory networks of cancer cells can generate survival dependencies on otherwise nonessential gene products (Lowe et al., 2004). We have previously identified a key role for RalB GTPase activity in the support of cell survival in the face of oncogenic stress (Chien and White, 2003) and now demonstrate that a RalB/Sec5/TBK1 activation complex represents the functional core of that support. We have not yet determined if there are specific prosurvival/antiapoptotic components of the altered gene expression programs induced by IRF-3 and IRF-7 in response to TBK1 activation or if additional TBK1 substrates remain to be identified that support cell survival. Of note, IRF-7 expression is sufficient to promote anchorage-independent proliferation and is significantly associated with the pathogenesis of EBV-associated lymphomas (Zhang et al., 2004).

In summary, we have defined the mechanistic contribution of RalB to oncogenic transformation and revealed the RalB/Sec5 effector complex as an obligatory component of the proximal machinery engaging TBK1 activation in response to antiviral surveillance signaling. The functional relationship of the RalB/Sec5/TBK1 activation complex to tumor cell survival suggests that oncogenic transformation commandeers cell-autonomous host defense signaling to deflect cell-death checkpoint activation. This relationship reveals a novel aspect of the aberrant cell regulatory programs supporting tumorigenicity and suggests proteins like TBK1 may be conceptually ideal candidate targets for development of drugs with a large therapeutic window.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection

All human cell lines were handled as previously described (Chien and White, 2003), except osteosarcoma cells were maintained in DMEM with 10% FBS and transfected with Fugene 6 (Roche) and HBECs were maintained in defined Keratinocyte-SFM medium (GIBCO). siRNAs were delivered using Oligofectamine (Life Technologies) in all cell lines. To obtain high-transfection efficiencies (>90%) of HMECs and HBECs, cultures were exposed to trypsin to induce macropinocytosis on apical and lateral surfaces (Chien and White, 2003).

Materials

Myc-RalF39L, HA-Sec5, Myc-RLIPrbd, Myc-Sec5-RBD, GST-RLIPrbd, and GST-Sec5rbd constructs are as previously described (Moskalenko et al., 2002). FLAG-TBK1 (WT), FLAG-TBK1 (K38A), and GST-IRF-3C′ (residues 375–427) were kindly provided by Dr. James Chen (UT Southwestern Medical Center). Synthetic siRNAs targeting RalA and RalB were as previously described (Chien and White, 2003). Synthetic siRNAs targeting Sec5, Sec6, Sec8, Sec10, Exo84, and TBK1 were designed by standard methods (see Supplemental Experimental Procedures in the Supplemental Data available with this article online). The following antibodies were used: mouse monoclonal anti-RalA and rabbit polyclonal anti-RalB (BD, Transduction Laboratories); rabbit polyclonal ERK1/2 (Santa Cruz Biotechnology); rabbit polyclonal anti-Sec5, mouse monoclonal anti-Sec6; rabbit polyclonal anti-Sec10, rabbit polyclonal anti-Exo84 (Moskalenko et al., 2002, 2003); rabbit polyclonal anti-IRF-3 (Santa Cruz Biotechnology); rabbit polyclonal anti-phospho-IRF-3 (Ser396) (Upstate); and mouse monoclonal anti-TBK1/NAK (Imgenex), mouse monoclonal anti-Ras (BD), and rabbit polyclonal anti-GFP (Santa Cruz Biotechnology). Phospho-specific antibodies were purchased from Cell Signaling. Poly(I:C) was reconstituted in PBS according to manufacturer's instruction (Amersham-Pharmacia). Sendai virus (Cantell strain, Charles River Laboratories) was used at a concentration of 100 (HA) units/ml. IFN-β production was measured using the human IFN-β ELISA (PBL Biomedical Laboratories). TUNEL was carried out according to manufacturer's instructions (BD, Biosciences).

Immunoprecipitation and Mass Spectrometry

Sec8-associated proteins were immunoprecipitated from 16 mg HeLa cytosolic extracts prepared according to Dignam et al. (1983). Proteins were eluted and separated by SDS-PAGE. Gels were stained with colloidal Coomassie blue (Invitrogen), and protein bands of interest were excised and in-gel digested. The resulting tryptic peptides were identified as previously described (Zhao et al., 2004b). For all other IPs, cells were lysed in 1% NP-40, 10 mM Tris (pH 7.6), 250 μM sodium deoxycholate, 1 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 150 mM NaCl, 50 mM NaF, 1 mM sodium vanadate, and 80 mM β-glycerol phosphate. Cleared supernatants were collected and incubated with antibody-conjugated agarose for 2 hr. Beads were then washed four times in lyses buffer plus 250 mM NaCl prior to immunoblot analysis.

In Vitro Binding and Protein Kinase Assays

Purified recombinant GST-RLIPrbd and GST-Sec5rbd were immobilized on glutathione-Sepharose beads. Ten micrograms of each GST fusion protein were incubated with 0.5 μg of recombinant TBK1 protein (Upstate) for 1 hr at 4°C. Beads were then wash four times with 50 mM Tris (pH7.6), 0.3 M NaCl, and 0.1% Triton X-100 prior to SDS-PAGE analysis. TBK1 I.P. kinase assays were as described (McWhirter et al., 2004).

Ral and Ras Activation Assays

Cells were lysed in 50 mM Tris (pH 7.6), 10% glycerol, 200 mM NaCl, 2.5 mM $MgCl_2$, and 1% triton X-100. Cleared supernatants were collected and incubated with GST-Sec5rbd or GST-Raf1rbd immobilized on glutathione-Sepharose beads for 1 hr at 4°C. Beads were washed three times with lysis buffer prior to SDS-PAGE analysis.

RT-PCR

Total RNA was isolated from HBEC cells using High Pure RNA Isolation Kit (Roche) and subjected to RT-PCR using SuperScript III One-Step RT-PCR system (Invitrogen) to measure expression of ISG56 and GAPDH. Gene-specific primer sequences are described in Supplemental Experimental Procedures. Quantitative analysis of *Drosophila* fat body *drs* expression was performed as previously described (Leulier et al., 2003).

Supplemental Data

Supplemental Data for this article include Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/cgi/content/full/127/1/157/DC1/.

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